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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(71) Applicant (for all designated States except US): GIST-BROCADES B.V. [NL/NL]; Wateringseweg 1, P.O. Box 1, NL-2600 MA Delft (NL).  (72) Inventors; and (75) Inventors/Applicants (for US only): BOVENBERG, Roelof, Ary, Lans [NL/NL]; 's-Gravenweg 121, NL-3062 ZD Rotterdam (NL); VAN DER LAAN, Jan, Metske [NL/NL]; Leursebaan 364, NL-4839 AP Breda (NL); KERKMAN, Richard [NL/NL]; Koninginneweg 12, NL-2042 NL Zandvoort (NL); NIEBOER, Maarten [NL/NL]; Gerberasingel 112, NL-2651 XZ Berkel en Rodenrijs (NL).  (74) Agents: VISSER-LUIRINK, Gesina et al.; Gist-Brocades N.V., Patents and Trademarks Dept., Wateringseweg 1, P.O. Box 1, NL-2600 MA Delft (NL).		Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.	
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(54) Title: PROCESS FOR THE PRODUCTION OF ADIPOYL CEPHALOSPORINS

(57) Abstract

An improved process for the preparation of adipoyl cephalosporins via enzymatic ring expansion of adipoyl-6-aminopenicillanic acid, using a *Penicillium chrysogenum* transformant strain expressing modified expandase enzyme.

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Attorney Docket No. 6653-019-999  
Reference AM

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 97/03879

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC 6	C12N15/52	C12N9/00	C12P35/02	C12N1/15
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According to International Patent Classification(IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CANTWELL C ET AL: "ISOLATION OF DEACETOXYCEPHALOSPORIN C FROM FERMENTATION BROTHS OF PENICILLIUM-CHRYSOGENUM TRANSFORMANTS CONSTRUCTION OF A NEW FUNGAL BIOSYNTHETIC PATHWAY" PROC R SOC LOND SER B BIOL SCI, 248 (1323). 1992. 283-289., XP002049879 cited in the application see the whole document ---	1-8
A	EP 0 341 892 A (LILLY CO ELI) 15 November 1989 cited in the application see claims 1-27 ---	1-8
A	EP 0 366 354 A (LILLY CO ELI) 2 May 1990 cited in the application see claims 1-10 ---	1-8
	-/--	



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Patent family members are listed in annex.

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**INTERNATIONAL SEARCH REPORT**

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**C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT**

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SKATRUD PL: "GENETIC-ENGINEERING OF BETA-LACTAM ANTIBIOTIC BIOSYNTHETIC PATHWAYS IN FILAMENTOUS FUNGI" TRENDS IN BIOTECHNOLOGY, 1992, 10, 324-329, XP002049880 see page 37, left-hand column, paragraph 3 - right-hand column, paragraph 1; figure 3 ---	1-8
A	CANTWELL C A ET AL: "CLONING AND EXPRESSION OF A HYBRID STREPTOMYCES CLAVULIGERUS CEFE GENE IN PENICILLIUM CHRYSOGENUM" CURRENT GENETICS, vol. 17, 1990, pages 213-221, XP000606288 see the whole document ---	1-8
A	BALDWIN J E ET AL: "GENETIC ENGINEERING OF CEPHALOSPORIN BIOSYNTHESIS" PROCEEDINGS OF THE INTERNATIONAL SYMPOSIUM ON THE GENETICS OF INDUSTRIAL MICROORGANISMS, 1994, page 184 XP000646621 see paragraph 1 ---	1-8
P,X	WO 97 20053 A (GIST BROCADES BV ;UNIV OXFORD (GB); SUTHERLAND JOHN DAVID (GB); B0) 5 June 1997 see the whole document -----	1,3-6

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(S1) International Patent Classification 6 : <b>C12N 15/52, C12P 35/00, C12N 9/00, 1/15</b>		A2	(11) International Publication Number: <b>WO 98/02551</b> (43) International Publication Date: 22 January 1998 (22.01.98)
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(74) Agents: VISSER-LUIRINK, Gesina et al.; Gist-Brocades N.V., Patents and Trademarks Dept., Wateringseweg 1, P.O. Box 1, NL-2600 MA Delft (NL).			
(54) Title: IMPROVED PROCESS FOR THE PRODUCTION OF ADIPOYL CEPHALOSPORINS			
(57) Abstract			
An improved process for the preparation of adipoyl cephalosporins via enzymatic ring expansion of adipoyl-6-aminopenicillanic acid, using a <i>Penicillium chrysogenum</i> transformant strain expressing modified expandase enzyme.			

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IMPROVED PROCESS FOR THE PRODUCTION OF  
ADIPOYL CEPHALOSPORINS

Field of the invention and brief description of the prior art

The present invention concerns a biosynthetic process for preparation and recovery of adipoyl cephalosporins (5-carboxypentanoyl cephalosporins). Adipoyl-7-aminocephalosporins include adipoyl-7-aminodesacetoxycephalosporanic acid, adipoyl-7-aminodesacetylcephalosporanic acid and adipoyl-7-aminocephalosporanic acid. The 7-aminocephalosporines which can be obtained after deacylation of the adipoyl cephalosporins, 7-aminodesacetoxycephalosporanic acid (7-ADCA), 7-aminodesacetylcephalosporanic acid or 7-aminocephalosporanic acid (7ACA) respectively, are key intermediates used in the preparation of semi-synthetic cephalosporins (SSC's).

$\beta$ -Lactam antibiotics constitute the most important group of antibiotic compounds, with a long history of clinical use. Among this group, the prominent ones are the penicillins and cephalosporins. These compounds are naturally produced by the filamentous fungi *Penicillium chrysogenum* and *Acremonium chrysogenum*, respectively.

As a result of classical strain improvement techniques, the production levels of the antibiotics in *Penicillium chrysogenum* and *Acremonium chrysogenum* have increased dramatically over the past decades. With the increasing knowledge of the biosynthetic pathways leading to penicillins and cephalosporins, and the advent of recombinant DNA technology, new tools for the improvement of production strains and for the *in vivo* derivatization of the compounds have become available.

Most enzymes involved in  $\beta$ -lactam biosynthesis have been identified and their corresponding genes been cloned, as can be found in Ingolia and Queener, Med. Res. Rev. 9

- 2 -

(1989), 245-264 (biosynthesis route and enzymes), and Aharonowitz, Cohen, and Martin, Ann. Rev. Microbiol. 46 (1992), 461-495 (gene cloning).

The first two steps in the biosynthesis of penicillin in *P. chrysogenum* are the condensation of the three amino acids L-5-amino-5-carboxypentanoic acid (L- $\alpha$ -amino adipic acid) (A), L-cysteine (C) and L-valine (V) into the tripeptide LLD-ACV, followed by cyclization of this tripeptide to form isopenicillin N. This compound contains the typical  $\beta$ -lactam structure.

The third step involves the exchange of the hydrophilic side chain of L-5-amino-5-carboxypentanoic acid by a hydrophobic side chain by the action of the enzyme acyltransferase (AT). The enzymatic exchange reaction mediated by AT takes place inside a cellular organelle, the microbody, as has been described in EP-A-0448180.

Cephalosporins are much more expensive than penicillins. One reason is that some cephalosporins (e.g. cephalexin) are made from penicillins by a number of chemical conversions. Another reason is that, so far, only cephalosporins with a D-5-amino adipoyl side chain could be fermented. Cephalosporin C, by far the most important starting material in this respect, is very soluble in water at any pH, thus implying lengthy and costly isolation processes using cumbersome and expensive column technology. Cephalosporin C obtained in this way has to be converted into therapeutically used cephalosporins by a number of chemical and enzymatic conversions.

The methods currently favoured in industry to prepare the intermediate 7-ADCA involve complex chemical steps leading to the expansion and derivatization of penicillin G. One of the necessary chemical steps to produce 7-ADCA involves the expansion of the characteristic 5-membered ring structure of penicillins to the typical 6-membered ring structure of cephalosporins (see for instance US 4,003,894). This complex chemical processing is both expensive and noxious to the environment.

Consequently, there is a great desire to replace such chemical processes with enzymatical reactions such as

enzymatic catalysis, preferably during fermentation. A key to the replacement of the chemical expansion process by a biological process is the central enzyme in the cephalosporin biosynthetic pathway, desacetoxycephalosporin C synthase (DAOCS), or expandase.

The expandase enzyme from the bacterium *Streptomyces clavuligerus* has been well characterized (EP-A-0366354) both biochemically and functionally, as has its corresponding gene. Both physical maps of the *cefE* gene (EP-A-0341892), DNA sequence and transformation studies in *P. chrysogenum* with *cefE* have been described. When introduced into *P. chrysogenum*, it can convert the penicillin ring structure into the cephalosporin ring structure, as described in Cantwell *et al.*, Proc. R. Soc. Lond. B. 248 (1992), 283-289.

Other sources for a ring expansion enzyme are the bacteria *Nocardia lactamdurans* (formerly *Streptomyces lactamdurans*) and *Lysobacter lactamgenus*. Both the biochemical properties of the enzyme and the DNA sequence of the gene have been described for *Nocardia lactamdurans* (Cortés *et al.*, J. Gen. Microbiol. 133 (1987), 3165-3174; and Coque *et al.*, Mol. Gen. Genet. 236 (1993), 453-458, respectively). For *Lysobacter lactamdurans* the gene cluster involved in cephalosporin biosynthesis was sequenced and sequences of several key enzymes were deposited to the EMBL Data Library (Kimura *et al.*, October 1990, entry code EMBL:X56660).

It has recently been found that the expandase enzyme is capable of expanding penicillins with particular side chains to the corresponding 7-ADCA derivative. This feature of the expandase has been exploited in the technology as disclosed in EP-A-0532341, WO95/04148 and WO95/04149. In these disclosures the conventional chemical conversion of penicillin G to 7-ADCA has been replaced by the *in vivo* conversion of certain 6-aminopenicillanic acid (6-APA) derivatives in recombinant *Penicillium chrysogenum* strains containing an expandase gene.

In EP-A-0532341 the application of an adipate (5-carboxypentanoate) feedstock has been disclosed. The incorporation of this substrate leads to a penicillin

derivative with an adipoyl side chain, viz. adipoyl-6-APA. This incorporation is due to the fact that the acyltransferase has a proven wide substrate specificity (Behrens *et al.*, J. Biol. Chem. 175 (1948), 751-809; Cole, Process. Biochem. 1 (1966), 334-338; Ballio *et al.*, Nature 185 (1960), 97-99).

More particularly, EP-A-0532341 teaches the *in vivo* use of the expandase enzyme in *P. chrysogenum*, in combination with a adipoyl side chain as a feedstock, which is used as a substrate for the acyltransferase enzyme in *P. chrysogenum*. This leads to the formation of adipoyl-6-APA, which is converted by an expandase enzyme introduced into the *P. chrysogenum* strain to yield adipoyl-7-ADCA. Finally, the removal of the 5-adipoyl side chain is suggested, yielding 7-ADCA as a final product. The patent application EP-A-0540210 describes a similar process for the preparation of 7-ACA, including the extra steps of converting the 3-methyl side chain of ADCA into the 3-acetoxyethyl side chain of ACA.

In WO95/04148 and WO95/04149 it has been disclosed that 3'-carboxymethylthiopropionic acid and 3,3'-thiodipropionic acid, respectively were found to be substrates for the expandase, yielding respectively 2-(carboxyethylthio)acetyl-7-ADCA and a mixture of 3-(carboxymethylthio)propionyl-7-ADCA and 2-(carboxyethylthio)acetyl-7-ADCA. In addition a process was described for the recovery of these cephalosporins from the fermentation broth and the subsequent removal of the side chains by an enzymatic process.

The alternative side chains which are provided above allow for the production of anionic cephalosporins instead of the conventional zwitterionic cephalosporins such as cefC. This allows for a more simple isolation procedure. In addition these side chains can be removed by an enzymatic process. As a consequence these alternative side chains can be regarded as ideal protecting groups for the 7 amino position of the cephalosporin. Due to their beneficial properties mentioned above, cephalosporins with these alternative side chains form a useful starting point for

chemical synthesis where it is required to protect the 7 amino position of the cephalosporin ring.

The observation that substantial quantities of desacetoxycephalosporin C (DAOC) can be formed by non-precursed *P. chrysogenum* transformants expressing expandase implies the presence of significant amounts of penicillin N, the natural substrate for expandase, in *P. chrysogenum* (Alvi *et al.* (1995), J. Antibiot. 48, p338-340). As a consequence, with a adipoyl side chain as feedstock, in the *P. chrysogenum* transformants which express expandase activity, penicillin N competes with adipoyl-6-APA for ring expansion resulting in substantial formation of  $\alpha$ -(D) amino adipoyl-7ADCA (DAOC) at the expense of desired product adipoyl-7-ADCA. In addition to the accumulation of  $\alpha$ -(D) amino adipoyl-7ADCA, part of the intermediate adipoyl-6-APA is excreted before ring expansion by expandase can occur. As a consequence of producing these by-products additional precautions have to be taken in order to remove these by-products during the recovery of adipoyl-7ADCA. Apart from recovery problems the production of these by-products is a significant waste of  $\beta$ -lactam producing capacity of the strains which ultimately limits the final yield of adipoyl-7ADCA. Redirecting of this  $\beta$ -lactam by-product waste stream into the main adipoyl-7-ADCA synthesis route would benefit the final fermentation yield of adipoyl cephalosporin with regard to yield as well as with regard to the quality of the product.

Recently, the structure of the isopenicillin N synthetase (IPNS) enzyme of *A. nidulans* (aIPNS) has been determined (Roach (1995), Nature, 375, p700-704). IPNS and expandase belong to the same family of oxidase enzymes. They share biochemical characteristics and, on the basis of sequence homologies, it has been proposed that structural similarities exist between the two enzymes (Roach *et al.*, *supra*; Cooper (1993), Bioorganic Med. Chem. 1, p1-17).

The mechanism of IPNS activity has been described in several reports (see for example: Blackburn *et al.* (1995), Biochemistry 34, p7548-7562). It is proposed, from an analysis of the chemistry catalysed by IPNS, that the

cysteinyl thiol group of ACV must bind to the ferrous ion at the active site in the enzyme-substrate complex. Given this implicit attachment point between the substrate and the enzyme a large number of conformationally distinct binding modes can be distinguished given the crystallographically determined constraints of the active site. It is therefore not obvious how ACV binds to aIPNS and, by inference, the mode of binding of penicillin N to expandase is even less apparent.

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#### Brief description of the figures

Figure 1: Sequence alignment of Isopenicillin N synthetases (IPN synthetases) with expandases (desacetoxycephalosporin C synthases or DAOCS) and cephalosporin 3' hydroxylases (desacetylcephalosporin C synthase or DACS). Listed are IPN synthetase *Aspergillus nidulans*, *Streptomyces clavuligerus*, *Streptomyces anulatus*, *Streptomyces lactamdurans*, *Flavobacterium* sp. (strain SC 12154), *Streptomyces griseus* (strain SC 12154), *Lysobacter lactamgenus*, *Streptomyces jumonjinensis*, *Streptomyces cattleya*, DAOCS of *Streptomyces clavuligerus*, DACS of *Streptomyces clavuligerus*, DACS of *Streptomyces lactamdurans*, DAOCS/DACS of *Cephalosporium acremonium*, DACS of *Lysobacter lactamgenus* (strain YK90), DACS of *Lysobacter lactamgenus* (strain YK90).

Figure 2: Schematic representation of plasmid pZEx.

Figure 3: Schematic representation of plasmid pZExD96N.

Figure 4: Schematic representation of plasmid pZExD96Q.

Figure 5: Schematic representation of plasmid pZExD96M.

Figure 6: Schematic representation of plasmid pZExD96K.

Figure 7: Schematic representation of plasmid pZExD96H.

#### Summary of the invention

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The present invention provides a more efficient process for the preparation and recovery of adipoyl cephalosporins by:

- a) transforming a *Penicillium chrysogenum* strain with an expandase gene encoding a modified expandase enzyme, under the transcriptional and translational regulation of fungal expression signals;
- b) fermenting said strain in a culture medium and adding to said culture medium adipic acid or a salt or ester thereof suitable to yield adipoyl-6-APA, which is expanded to form adipoyl-7-ADCA;
- c) recovering the adipoyl-7-ADCA from the fermentation broth;
- d) deacylating adipoyl-7-ADCA; and
- e) recovering the crystalline 7-ADCA.

In particular the process exhibits a better efficiency because the production of adipoyl-7ADCA is improved relative to production of the main by-products  $\alpha$ -D-amino adipoyl-7-ADCA (DAOC) and adipoyl-6-APA.

Preferably, adipoyl-7-ADCA is recovered from the fermentation broth by extracting the broth filtrate with an organic solvent immiscible with water at a pH of lower than about 4.5 and back-extracting the same with water at a pH between 4 and 10.

Moreover, the DNA encoding modified expandase and a recombinant DNA vector comprising the same, functionally linked to the transcriptional and translational control elements of a fungal gene, for instance *Aspergillus nidulans* *gpdA* gene, and the *P. chrysogenum* *pcbC* gene and host cells transformed with the same, are provided.

#### Detailed description of the invention

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The present invention concerns the use of functional gene constructs encoding modified expandase enzyme in *P. chrysogenum* for the *in vivo* expansion of the adipoyl-6-APA to form the adipic acid derivative of a key intermediate in the cephalosporin biosynthesis, 7-aminodesacetoxycephalosporanic acid, or 7-ADCA. This derivative has a chemical composition so as to allow efficient solvent extraction, thus providing an economically attractive recovery process.

Modification of the expandase gene is directed at producing expandase mutants which best expand adipoyl-6-APA in *in vitro* and/or *in vivo* context where other penicillins such as penicillin N and isopenicillin N can act as competing substrates. This is an essential feature of the invention given the observation of significant amounts of penicillin N being produced by *P. chrysogenum* and the knowledge that penicillin N is a significantly better substrate than adipoyl-6-APA for the wildtype expandase. By transforming *P. chrysogenum* with such targeted mutants of expandase, novel *P. chrysogenum* strains can be obtained which have an improved capacity for the production of adipoyl-7-ADCA.

The ring expansion of adipoyl-6-APA is a key step in the production of adipoyl cephalosporins. In *P. chrysogenum* strains which are only transformed with the expandase gene, adipoyl-7-ADCA is the end product of the fermentation. When in addition *P. chrysogenum* expresses deacetylcephalosporin C synthase (DACS; the *cefF* gene in *Streptomyces*, the *cefEF* gene in *Acremonium*) as well then adipoyl-7-desacetyl-cephalosporanic acid is the end product. When finally also desacetylcephalosporin C acetyltransferase (the *cefG* gene) is expressed then adipoyl-7-ACA is produced. More efficient production of adipoyl-7-ADCA will also improve production of the other adipoyl-cephalosporins.

Transformation of *P. chrysogenum* can, in principle, be achieved by different means of DNA delivery, like PEG-Ca mediated protoplast uptake, electroporation or particle gun techniques, and selection of transformants. See for example Van den Hondel en Punt, Gene and Transfer and Vector Development for Filamentous Fungi, in: Applied Molecular Genetics of Fungi (Peberdy, Laten, Ogden, Bennett, eds.), Cambridge University Press (1991). The application of dominant and non-dominant selection markers has been described (Van den Hondel, supra). Selection markers of both homologous (*P. chrysogenum* derived) and heterologous (non-*P. chrysogenum* derived) origin have been described (Gouka *et al.*, J. Biotechnol. 20 (1991), 189-200).

The application of the different transformant selection markers, homologous or heterologous, in the presence or absence of vector sequences, physically linked or not to the non-selectable DNA, in the selection of transformants are well known.

The ring-expansion reaction, mediated by the modified expandase enzyme is introduced into and expressed in this way in *P. chrysogenum*, for instance in strain Wisconsin 54-1255 (deposited at ATCC under accession number 28089). Other strains of *P. chrysogenum*, including mutants of strain Wisconsin 54-1255, having an improved beta-lactam yield, are also suitable.

Furthermore, the modified *cefE* gene is placed under the transcriptional and translational control of fungal (be they filamentous or not) gene control elements. Those elements can be obtained from cloned fungal genes like the *P.chrysogenum* IPNS gene, the  $\beta$  tubulin gene, the *Aspergillus nidulans* *gpdA* gene, or the *Aspergillus niger* *glcA* gene.

In summary, the present invention teaches how the activity of a modified expandase enzyme expressed by a mutated gene which is introduced into *P. chrysogenum*, can be used to improve the yield of adipoyl cephalosporins resulting from the *in vivo* ring expansion of adipoyl-6-APA.

In accordance with the present invention the  $\beta$ -lactam intermediate adipoyl-7-ADCA is produced in *P. chrysogenum* by adding adipic acid or a salt or an ester thereof to the medium. Suitable salts are for instance those of sodium or potassium. Adipoyl-7-ADCA is efficiently recovered from the medium through a simple solvent extraction, for instance, as follows:

The broth is filtered and an organic solvent immiscible with water is added to the filtrate. The pH is adjusted in order to extract the cephalosporin from the aqueous layer. The pH range has to be lower than 4.5; preferably between 4 and 1, more preferably between 2 and 1. In this way the cephalosporin is separated from many other impurities present in the fermentation broth. Preferably a small volume of organic solvent is used, giving a more concentrated solution of the cephalosporin, so achieving

reduction of the volumetric flow rates. A second possibility is whole broth extraction at a pH of 4 or lower. Preferably the broth is extracted between 4 and 1 with an organic solvent immiscible with water.

Any solvent that does not interfere with the cephalosporin molecule can be used. Suitable solvents are, for instance, butyl acetate, ethyl acetate, methyl isobutyl ketone, alcohols like butanol etc.. Preferably 1-butanol or isobutanol are used.

Hereafter the cephalosporin is back extracted with water at a pH between 4 and 10, preferably between 6 and 9. Again the final volume can be reduced. The recovery can be carried out at temperatures between 0 and 50°C, and preferably at ambient temperatures.

The aqueous cephalosporin solution thus obtained is treated with a suitable enzyme in order to remove the adipoyl side chain and obtain the desired 7-ADCA.

Preferably, an immobilized enzyme is used, in order to be able to use the enzyme repeatedly. The methodology for the preparation of such particles and the immobilization of the enzymes have been described extensively in EP-A-0222462. The pH of the aqueous solution has a value of, for example pH 4 to pH 9, at which the degradation reaction of cephalosporin is minimized and the desired conversion with the enzyme is optimized. Thus, the enzyme is added to the aqueous cephalosporin solution while maintaining the pH at the appropriate level by, for instance, adding an inorganic base, such as a potassium hydroxide solution, or applying a cation exchange resin. When the reaction is completed the immobilized enzyme is removed by filtration. Another possibility is the application of the immobilized enzyme in a fixed or fluidized bed column, or using the enzyme in solution and removing the products by membrane filtration. Subsequently, the reaction mixture is acidified in the presence of an organic solvent immiscible with water.

Suitable enzymes are, for instance, derived from a *Pseudomonas* SY77 microorganism having a mutation in one or more of the positions 62, 177, 178 and 179. Also enzymes from other *Pseudomonas* microorganisms, preferably

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*Pseudomonas* SE83, optionally having a mutation in one or more of the positions corresponding to the 62, 177, 178 and 179 positions in *Pseudomonas* SY77, may be used.

After adjusting the pH to about 0.1 to 1.5, the layers are separated and the pH of the aqueous layer is adjusted between 2 and 5, more preferably between 3 and 4. The crystalline 7-ADCA is then filtered off.

The deacylation can also be carried out chemically as known in the prior art, for instance, via the formation of an iminochloride side chain, by adding phosphorus pentachloride at a temperature of lower than 10°C and subsequently isobutanol at ambient temperatures or lower.

The following examples are offered by way of illustration and not by way of limitation. The overall approach entails i) identification of residues of expandase involved in substrate specificity, ii) construction of mutant expandase proteins, iii) subcloning of mutant expandase genes in *P. chrysogenum* expression vectors and expression of the mutant expandase in *P. chrysogenum*, iv) determination of the adipoyl-7-ADCA production versus production of  $\alpha$ -D-amino adipoyl-7-ADCA and adipoyl-6-APA.

In a similar way as has been described for the adipoyl side chain a person skilled in the art may also adapt the expandase enzyme towards the processes as have been disclosed in WO95/04148 and WO95/04149 which use 3'-carboxy-methylthiopropionic acid and 3,3'-thiodipropionic acid as side chains, yielding 2-(carboxyethylthio)acetyl-7-ADCA and a mixture of 3-(carboxymethylthio)propionyl-7-ADCA and 2-(carboxyethylthio)acetyl-7-ADCA respectively.

#### Example 1

**Identification of residues involved in the binding of the  $\alpha$ -amino group of the adipoyl side chain.**

Central to the invention is the proposal that, in the case of aIPNS, upon ACV binding, the L- $\alpha$ -amino adipoyl side chain of ACV displaces the C-terminal tail of the enzyme (glutamine 330, threonine 331 and a number of preceding residues) by virtue of the similarity between the L- $\alpha$ -

amino adipoyl side chain of ACV and the C-terminal dipeptide in steric and electronic terms. Comparison of the C-terminal tail and ACV reveals the similarity between the L- $\alpha$ -amino adipoyl side-chain of ACV the glutaminyl-threonine end 5 of the tail; specifically the carboxylates in both cases are functionally homologous. The relatedness of expandase to aIPNS suggests that the D- $\alpha$ -amino adipoyl side chain of the substrate penicillin N binds in a similar fashion to expandase as does the L- $\alpha$ -amino adipoyl side chain of ACV to 10 aIPNS. At the heart of the invention is the proposal that the D- $\alpha$ -amino adipoyl side chain of penicillin N will be bound by amino acid residues of expandase that are homologous to the amino acid residues of aIPNS involved in binding the L- $\alpha$ -amino adipoyl side chain of ACV.

15 We propose the  $\alpha$ -carboxyl group of the  $\alpha$ -amino adipoyl moiety as the major determinant in the substrate for binding to both aIPNS and expandase. As a consequence it is likely that the binding site for this carboxyl group is conserved between aIPNS and expandase. Expandase is strictly selective 20 for the D-enantiomer of the  $\alpha$ -amino adipoyl moiety, resulting in the exclusive expansion of Penicillin N. The same preference for the D-enantiomer holds for the desacetylcephalosporin C synthases (DACS) which show a high degree of homology with the expandases. In the cyclization 25 of the tripeptide LLD-ACV the L-enantiomer of  $\alpha$ -amino adipoyl moiety is converted by aIPNS. However aIPNS is not very selective and can also convert an ACV tripeptide which contains the D-enantiomer of the  $\alpha$ -amino adipoyl side chain. As a consequence we propose that the binding site for the  $\alpha$ - 30 carboxyl group of the  $\alpha$ -amino adipoyl side chain is conserved between the IPNS family and the expandase/hydroxylase family. The binding site of the  $\alpha$ -amino group is expected to be conserved quite strictly within the group of expandases/hydroxylases, but less well between the IPN 35 synthases and the expandases/hydroxylases.

In general positively charged aminogroups in the substrate are often accommodated by negatively charged residues in the protein. Therefore we aligned the expandases/hydroxylases (DAOCS/DACS) which are known at

present (Figure 1). There are 12 positions which exhibit complete conservation of a negative charge. Comparing these positions with the corresponding positions in the IPN synthetases reveals that only 5 of these positions also contain a strictly conserved negative charge in IPN synthetases. From the other positions which do not maintain a negative charge in IPN synthetases only the position in expandase, which corresponds to Asparagine 109 in aIPNS, is close enough to the proposed binding site of the  $\alpha$ -amino adipoyl side chain to contribute to the specific binding of the  $\alpha$ -amino group. Deletion of the negative charge in the expandases at the position corresponding to position 109 in aIPNS will decrease the specificity for the  $\alpha$ -amino group of the  $\alpha$ -D-amino adipoyl side chain (penicillin N) relative to the adipoyl moiety (adipoyl-6-APA) which does not contain the amino group. Deletion of the negative charge in expandase can be performed by site-directed mutagenesis. Substitution of the negative charge at the position corresponding with aIPNS 109 will alter the relative binding of penicillin N and adipoyl-6-APA to expandase in the ground state and subsequent intermediates and transition states for the expansion of these penicillins to DAOC and adipoyl-7-ADCA, respectively. Mutations at the aforementioned position of expandase will increase the expansion of adipoyl-7-ADCA, decrease the expansion of penicillin N and/or increase the relative ratio of adipoyl-7-ADCA to penicillin N expansion in a competitive scenario. This will result in an improved production process with an improved product/by-product ratio. Mutations are chosen in which the negative charge on position 109 is neutralized, or exchanged by a positively charged residue. Taking into regard the aspect that the mutations have to be accommodated by the structure without too many additional adaptations the following substitutions are preferred : D96N, D96Q, D96M, D96K, D96H (position 96 in *S. clavuligerus* corresponds with position 109 in aIPNS).

In order to improve adipoyl-6-APA as an isolated substrate it is necessary to improve  $V_{max}$  and, in a context where the concentration of adipoyl-6-APA is non-saturating, to lower the  $K_m$ . This is not only the case when adipoyl-6-

APA is an isolated substrate but also when adipoyl-6-APA is a substrate in the presence of other penicillins, in the first place penicillin N but also isopenicillin N. The relative and absolute amounts of each penicillin expanded 5 depend on the ratio of the individual rates which can be broken down into an equation of the form:

$$\frac{v^{\text{adipoyl-6-APA}}}{v^{\text{penicillin N}}} = \frac{V_{\max}^{\text{adipoyl-6-APA}} * K_m^{\text{penicillin N}} * [\text{adipoyl-6-APA}]}{V_{\max}^{\text{penicillin N}} * K_m^{\text{adipoyl-6-APA}} * [\text{penicillin N}]}$$

10 where  $V_{\max}$  corresponds to the maximum enzyme velocities,  $K_m$  is the Michaelis constant, and  $[\text{adipoyl-6-APA}]$  and  $[\text{penicillin N}]$  are the concentrations of adipoyl-6-APA and penicillin N respectively. Mutations at positions of the expandase listed below which result in an increase of the 15 ratio of  $V_{\max}^{\text{adipoyl-6-APA}} : V_{\max}^{\text{penicillin N}}$  are part of the invention. The specificity changes required can result from any single or multiple mutant that has values of  $V_{\max}$  and/or  $K_m$  for either or both substrates altered in any way such as to increase the ratio of  $V_{\max}^{\text{adipoyl-6-APA}} : V_{\max}^{\text{penicillin N}}$  in vitro or the 20 relative yield of adipoyl-7-ADCA compared to DAOC from a adipic acid precursed fermentation of a strain of *P. chrysogenum* transformed with the mutant *cefE* gene.

Based on the proposal that the adipoyl carboxylgroup is 25 accommodated by positions which correspond to positions Arg87 and Ser183 in combination with our proposal that a negative charge in expandase at the position corresponding with aIPNS 109 is important for binding of the  $\alpha$  amino group, a number of positions can be derived from the 30 structural model which affect the specificity for the adipoyl side chain with respect to the  $\alpha$ -amino adipoyl side chain of penicillin N.

Residues of *Streptomyces clavuligerus* expandase so 35 identified include, but are not restricted to:  
Phenylalanine 152 (homologous to Threonine 180 of aIPNS),

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Leucine 153 (homologous to Leucine 181 of aIPNS),  
Serine 187 (homologous to Serine 218),  
Arginine 266 (homologous to Asn 287 of aIPNS),  
Isoleucine 298 (homologous to Leucine 317 of aIPNS),  
Asparagine 301 (homologous to Glycine 320 of aIPNS),  
Tyrosine 302 (homologous to Leucine 321 of aIPNS),  
Valine 303 (homologous to Valine 322 of aIPNS).

- Mutation of these residues individually or in combination  
will alter the relative binding of penicillin N and adipoyl-  
6-APA to expandase in the ground state and subsequent  
intermediates and transition states for the expansion of  
these penicillins to DAOC and phenylacetyldesace-  
toxycephalosporin, respectively. Mutations at the  
aforementioned positions of expandase will increase the  
expansion of adipoyl-6-APA, decrease the expansion of  
penicillin N and/or increase the relative ratio of adipoyl-  
6-APA to penicillin expansion in a competitive scenario.
- In *S.clavuligerus* position 109 is located at the start of a  
long loop which connects a  $\beta$ -strand and an  $\alpha$ -helix. This loop  
covers the  $\alpha$ -amino adipoyl side chain. Modification of this  
loop adapts the specificity expandase towards other  
substrates. Modification of this loop includes substitution  
of one or more amino acids, insertions and deletions.

#### Example 2

##### Shifting the substrate specificity of expandase by exchanging Asp96

Mutations at position 96 are chosen which change the negative charge at this position. The charge is neutralized, or exchanged by a positively charged residue. The following mutants are described: D96N, D96Q, D96M, D96K, and D96H.

- a) General gene cloning and gene transformation procedures:  
Common techniques used in gene cloning procedures are used in the present application. These techniques include polymerase chain reactions (PCR), synthetic oligonucleotide

synthesis, nucleotide sequence analysis, enzymatic ligation and restriction of DNA, *E. coli* vector subcloning, transformation, and transformant selection, isolation and purification of DNA. These techniques are all very well known in the art and adequately described in many references. See for example Sambrook *et al.*, Molecular Cloning, a Laboratory Manual, Cold Spring Harbor, U.S.A. (1989), Innes *et al.*, PCR protocols, a Guide to Methods and Applications, Academic Press (1990), and McPherson *et al.*, PCR, a Practical Approach, IRL Press (1991).

General procedures used in transformation of filamentous fungi and transformant selection include preparation of fungal protoplasts, DNA transfer and protoplast regeneration conditions, transformant purification and characterization. These procedures are all known in the art and very well documented in: Finkelstein and Ball (eds.), Biotechnology of Filamentous Fungi, technology and products, Butterworth-Heinemann (1992); Bennett and Lasure (eds.), More Gene Manipulations in Fungi, Academic Press (1991); Turner, in: Pühler (ed.), Biotechnology, second completely revised edition, VCH (1992).

More specific applications of gene cloning and gene transformation technology to *Penicillium chrysogenum* are well documented in Bennett and Lasure (supra), Finkelstein and Ball (supra), and EP 0 357 119.

b) Construction of mutants on Asp96:

The expandase expression cassette pZEx, which contains the wild type *Streptomyces clavuligerus* expandase gene including the IPNS promoter and AT terminator, is constructed as described below. The *S. clavuligerus* expandase gene including the AT terminator is derived from plasmid pASEWA (described in WO 95/04149). pASEWA is cut with *NdeI/NotI*, and the expandase-AT terminator fragment is isolated. The IPNS promoter is derived from *P. chrysogenum* chromosomal DNA in a PCR reaction using primers pcrA and pcrB (Table I), which are designed based on the IPNS promoter sequence (Smith *et al.* (1990), EMBO J. 9, p2743-

2750). The 0.9 kb PCR fragment is cut with *NdeI/NotI*, and the expandase- AT terminator fragment and the IPNS promoter fragment are ligated and inserted into the *NotI* site of pZErO (Invitrogen). Plasmid pZEx (Figure 2) is identified by restriction mapping.

The different expandase 96 mutants are constructed as follows: oligonucleotides (40-60 bases) are designed that cover the gene region between the *NdeI* site and the downstream *SacII* site in the expandase gene (see Figure 1).

10 The oligonucleotides have the following characteristics:

1. the *EcoNI* site is removed (oligonucleotides p2 and p8)
2. the upstream *SacII* site is removed (oligonucleotides p4 and p11)
3. the nucleotide sequence in p5 and p12 is varied in order

15 to make the mutations at D96.

pZExD96N (Figure 3): oligonucleotides p1, p2, p3, p4, p5(N), p6, p7, p8, p9, p10, p11, and p12(N) (Table I) are annealed and ligated. The double stranded DNA molecules are amplified by PCR, using primers pcr1 and pcr12 (Table I). The

20 resulting DNA fragment is cut with *NdeI* and *SacII*. pZEx is digested with these same enzymes, and mixed with the digested DNA fragment with the D96N mutation. After

25 ligation, the plasmid DNA is cut with *EcoNI* and introduced into *E. coli* TOP10F. Plasmid pZExD96N is identified by restriction mapping using *EcoNI* and *SacII*, and the presence of the mutation at amino acid position 96 is confirmed by nucleotide sequence analysis.

pZEx-D96Q (Figure 4): this plasmid is constructed as described for pZExD96N, except that oligonucleotides p5(Q) and p12(Q) are used instead of p5(N) and p12(N), respectively (Table I).

pZEx-D96M (Figure 5): this plasmid is constructed as described for pZExD96N, except that oligonucleotides p5(M) and p12(M) are used instead of p5(N) and p12(N), respectively (Table I).

pZEx-D96K (Figure 6): this plasmid is constructed as described for pZExD96N, except that oligonucleotides p5(K) and p12(K) are used instead of p5(N) and p12(N), respectively (Table I).

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- are purified by repeated cultivation on selective medium. Single stable colonies are used for further screening on the presence and expression of expandase by measuring the capacity of the transformants to produce cephalosporins.
- 5 Transformants are used to inoculate liquid medium as described in WO 95/04149, supplemented with 0.5-3 mg/ml sodium adipate as a side chain precursor for production tests. Filtrates of well grown cultures are analyzed by HPLC and NMR for production of adipoylcephalosporins and amino-
- 10 15 adipoylcephalosporins. Transformations with favourable adipoyl- over amino-adipoylcephalosporin production are selected.

- 20 -

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5

## (i) APPLICANT:

- (A) NAME: Gist-brocades
- (B) STREET: Wateringseweg 1
- (C) CITY: Delft
- 10 (E) COUNTRY: Netherlands
- (F) POSTAL CODE (ZIP): 2311 XT

15 (ii) TITLE OF INVENTION: Improved Process for the Production of  
Adipoyl Cephalosporins

15

(iii) NUMBER OF SEQUENCES: 24

## (iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- 20 (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

## 25 (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- 30 (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

35

## (vi) ORIGINAL SOURCE:

- (C) INDIVIDUAL ISOLATE: pcrl

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

40 GTTCGTAACA TATGGACACG ACGG

24

## (2) INFORMATION FOR SEQ ID NO: 2:

45

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs

- 21 -

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: DNA (synthetic)

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p2

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TGCACCAAGA CGAGTTCCGC AGGTGTCTGA GGGACAAGGG CCTCTTCTAT CTGACGGACT  
60

15

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 60 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: DNA (synthetic)

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p3

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GCGGTCTGAC CGACACCGAG CTGAAGTCGG CCAAGGACAT CGTCATCGAC TTCTTCGAGC  
60

35 (2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 60 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: DNA (synthetic)

45 (vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p4

- 22 -

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

ACGGCAGCGA GGCAGGAGAAG CGCGCCGTCA CCTCGCCCGT CCCCCACCATG CGACGCAGCT  
60

5

## (2) INFORMATION FOR SEQ ID NO: 5:

## (i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 60 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: DNA (synthetic)

## (vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p5(N)

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TCACCGGGCT GGAGTCGGAG AGCACCGCCC AGATCACCAA TACCGGCAGC TACTCCA  
60 ACT

25

## (2) INFORMATION FOR SEQ ID NO: 6:

## (i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 60 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: DNA (synthetic)

## (vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p5(Q)

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TCACCGGGCT GGAGTCGGAG AGCACCGCCC AGATCACCAA TACCGGCAGC TACTCCCAGT  
60

45 (2) INFORMATION FOR SEQ ID NO: 7:

## (i) SEQUENCE CHARACTERISTICS:

- 23 -

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: DNA (synthetic)

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p5(M)

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TCACCCGGGCT GGAGTCGGAG AGCACCGCCC AGATCACCAA TACCGGCAGC TACTCCATGT  
60

15

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: DNA (synthetic)

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p5(K)

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TCACCCGGGCT GGAGTCGGAG AGCACCGCCC AGATCACCAA TACCGGCAGC TACTCCAAGT  
60

35

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

45

(ii) MOLECULE TYPE: DNA (synthetic)

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p5(H)

- 24 -

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TCACCGGGCT GGAGTCGGAG AGCACCGCCC AGATCACCAA TACCGGCAGC TACTCCCACT  
60

5

## (2) INFORMATION FOR SEQ ID NO: 10:

## (i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 40 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: DNA (synthetic)

## (vi) ORIGINAL SOURCE:

- (C) INDIVIDUAL ISOLATE: p6

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

ACTCGATGTG CTACTCGATG GGCACCGCGG ACAACCTCTT  
40

25

## (2) INFORMATION FOR SEQ ID NO: 11:

## (i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 40 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: DNA (synthetic)

## (vi) ORIGINAL SOURCE:

- (C) INDIVIDUAL ISOLATE: p7

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

AGGCTGAAGG TGGGCACCGT CGTGTCCATA TGTTACGAAC  
40

45 (2) INFORMATION FOR SEQ ID NO: 12:

## (i) SEQUENCE CHARACTERISTICS:

- 25 -

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: DNA (synthetic)

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p8

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CCCTTGTCCC TCAGACACCT GCGGAACTCG TCTTGGTGCA GGCCCTGCTG GAGTTCGGCC  
60

15

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

- 20
- (A) LENGTH: 60 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: DNA (synthetic)

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p9

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

ATGTCCTTGG CCGACTTCAG CTCGGTGTAG GTCAGACCGC AGTCCGTCAG ATAGAAGAGGG  
60

35

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

- 40
- (A) LENGTH: 60 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

45

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: p10

- 26 -

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

ACGGGGCGAGG TGACGGCGCG CTTCTCCGCC TCGCTGCCGT GCTCGAAGAA GTCGATGACG  
60

5

## (2) INFORMATION FOR SEQ ID NO: 15:

## (i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 60 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: DNA (synthetic)

## (vi) ORIGINAL SOURCE:

- (C) INDIVIDUAL ISOLATE: p11

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

TTGGTGATCT GGGCGGTGCT CTCCGACTCC AGCCCGGTGA AGCCGCGTCG CATGGTGGGG  
60

25

## (2) INFORMATION FOR SEQ ID NO: 16:

## (i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 60 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: DNA (synthetic)

## (vi) ORIGINAL SOURCE:

- (C) INDIVIDUAL ISOLATE: p12(N)

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

AAGAGGTTGT CCGCGGTGCC CATCGAGTAG CACATCGAGT AGTTGGAGTA GCTGCCGGTA  
60

45 (2) INFORMATION FOR SEQ ID NO: 17:

## (i) SEQUENCE CHARACTERISTICS:

- 27 -

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: DNA (synthetic)

(vi) ORIGINAL SOURCE:

- (C) INDIVIDUAL ISOLATE: p12(Q)

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

AAGAGGTTGT CCGCGGTGCC CATCGAGTAG CACATCGAGT ACTGGGAGTA GCTGCCGGTA  
60

15

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: DNA (synthetic)

(vi) ORIGINAL SOURCE:

- (C) INDIVIDUAL ISOLATE: p12(M)

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

AAGAGGTTGT CCGCGGTGCC CATCGAGTAG CACATCGAGT ACATGGAGTA GCTGCCGGTA  
60

35

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

45

(ii) MOLECULE TYPE: DNA (synthetic)

(vi) ORIGINAL SOURCE:

- (C) INDIVIDUAL ISOLATE: p12(K)

- 28 -

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

AAGAGGTTGT CCGCGGTGCC CATCGAGTAG CACATCGAGT ACTTGGAGTA GCTGCCGGTA  
60

5

## (2) INFORMATION FOR SEQ ID NO: 20:

## (i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 60 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: DNA (synthetic)

## (vi) ORIGINAL SOURCE:

- (C) INDIVIDUAL ISOLATE: p12(H)

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

AAGAGGTTGT CCGCGGTGCC CATCGAGTAG CACATCGAGT ACTGGGAGTA GCTGCCGGTA  
60

25

## (2) INFORMATION FOR SEQ ID NO: 21:

## (i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 60 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: DNA (synthetic)

## (vi) ORIGINAL SOURCE:

- (C) INDIVIDUAL ISOLATE: p1

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

GTTCGTAACA TATGGACACG ACGGTGCCCA CCTTCAGCCT GGCCGAACTC CAGCAGGGCC  
60

45 (2) INFORMATION FOR SEQ ID NO: 22:

## (i) SEQUENCE CHARACTERISTICS:

- 29 -

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: DNA (synthetic)

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: pcr12

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

AAGAGGTTGT CCGCGGTGCC CATC

24

15

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: DNA (synthetic)

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: pcrA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

CGTCTGGATC GCGGCCGCCT TATACTGGGC CTGCTGCATT G

41

35

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

45

(ii) MOLECULE TYPE: DNA (synthetic)

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: pcrB

- 30 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

CGTCTGGATC CATATGGGTG TCTAGAAAAA TAATGGTG  
38

## Claims

- 5        1. A modified expandase gene encoding a mutant expandase which mutant expandase comprises:
- a) a substitution at one or more selected sites corresponding to a residue position selected from the group consisting of Aspartic acid 96, Phenylalanine 152, Leucine 153, Serine 187, Arginine 266, Isoleucine 298, Asparagine 301, Tyrosine 302, Valine 303 in *S.clavuligerus* expandase,
- 10      b) related to said wildtype expandase, an altered substrate specificity.
- 15        2. A modified expandase gene encoding a mutant expandase according to claim 1 which mutant expandase comprises one or more mutations selected from the group consisting of (a) D96N; (b) D96Q; (c) D96M; (d) D96K; (e) D96H.
- 20        3. An expression vector which comprises a modified expandase gene as defined in claim 1 or 2.
- 25        4. A microorganism host strain transformed with an expression vector as defined in claim 3.
- 30        5. An improved process for the preparation and recovery of 7-aminodesacetoxycephalosporanic acid (7-ADCA) by:
- a) transforming a *Penicillium chrysogenum* strain with a modified expandase gene as defined in claim 1 or 2, under the transcriptional and translational regulation of fungal expression signals;
- b) fermenting said strain in a culture medium and adding to said culture medium adipic acid or a salt or ester thereof suitable to yield adipoyl-6-APA, which is expanded to form adipoyl-7-ADCA;
- c) recovering the adipoyl-7-ADCA from the fermentation broth;

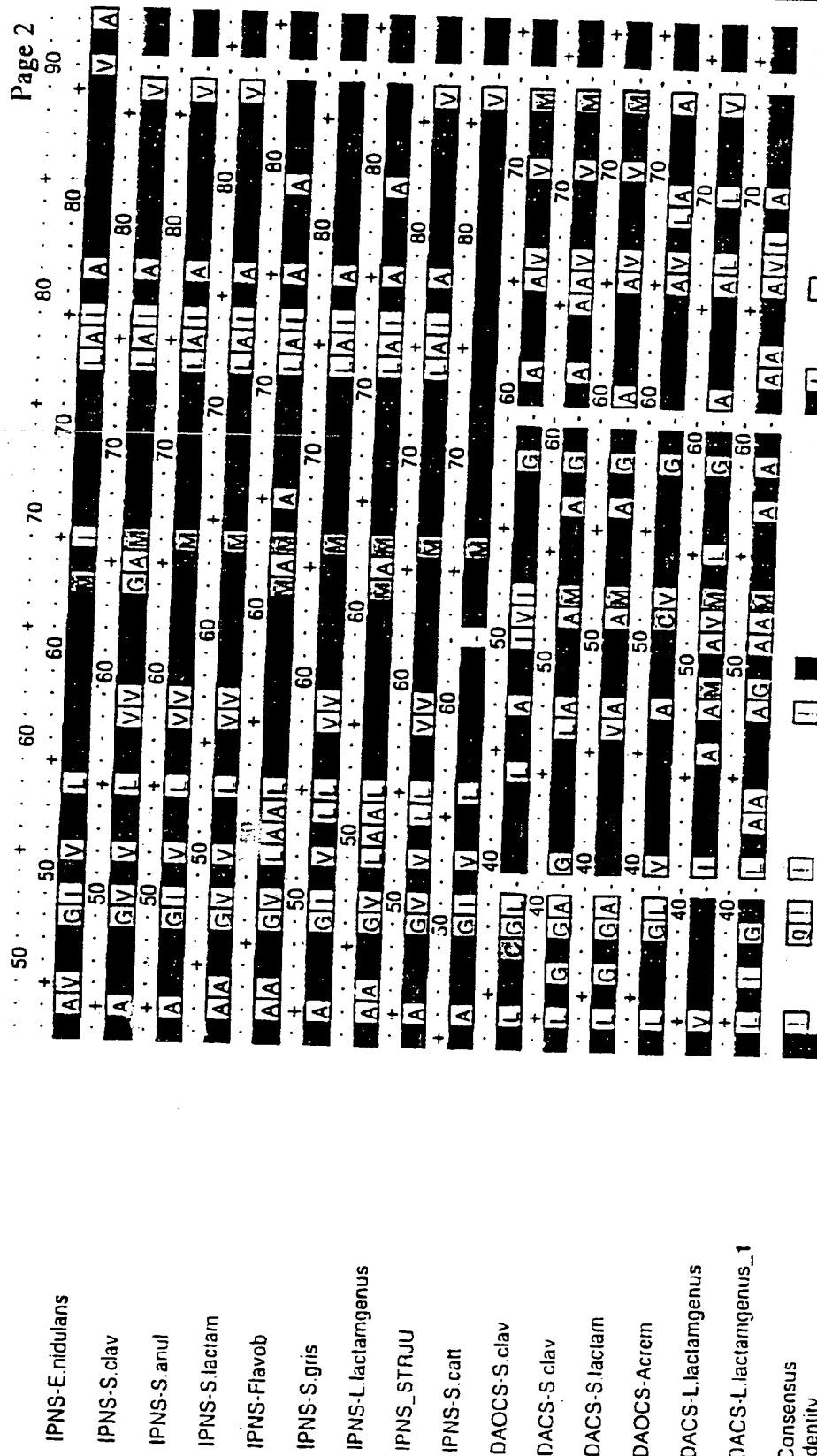
- d) deacylating adipoyl-7-ADCA; and
- e) recovering the crystalline 7-ADCA.

6. A process according to claim 5, wherein step (e) is  
a filtration step.

7. A process according to claim 5 or 6, wherein step  
(c) is a filtration step, and by extracting the broth  
filtrate with an organic solvent immiscible with water at a  
10 pH of lower than about 4.5 and back-extracting the same with  
water at a pH between 4 and 10.

8. A process according to anyone of the claims 5, 6 or  
7 wherein the expandase gene is derived from *Streptomyces*  
15 *clavuligerus* or *Nocardia lactamdurans* or *Lysobacter*  
*lactamgenus*.

Page 1



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Page 3

IPNS_E.nidulans	G	R	I	G	AIV	E	I	A	V	V
IPNS_S.clav	90	-	-	100	-	-	110	-	120	-
IPNS_S.anul	90	-	-	100	-	-	110	-	120	-
IPNS_S.lactam	90	-	-	100	-	-	110	-	120	-
IPNS_Flavob	90	-	-	100	-	-	110	-	120	-
IPNS_S.gris	90	-	-	100	-	-	110	-	120	-
IPNS_L.lactamgenus	90	-	-	100	-	-	110	-	120	-
IPNS_STRIJU	90	-	-	100	-	-	110	-	120	-
IPNS_S.catl	90	-	-	100	-	-	110	-	120	-
DAOCS_S.clav	80	-	-	90	-	-	100	-	110	-
DACS_S.clactam	80	-	-	90	-	-	100	-	110	-
DACS_S.clav	80	-	-	90	-	-	100	-	110	-
DAOCs_L.lactamgenus	80	-	-	90	-	-	100	-	110	-
DACS_L.lactamgenus_1	80	-	-	90	-	-	100	-	110	-

Consensus Identity

Page 4

IPNS-E.nidulans

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consensus

Page 5

	190	200	210	220	230
IPNS-E.nidulans	[A] V [V]	[A] V [V]	[A] A [A]	[A] G [G]	[V] [V]
IPNS-S.clav	[L] V [L]	[L] V [L]	[V] G [G]	[G] L [L]	[L] V [M]
IPNS-S.anul	[L] G [L]	[L] M [L]	[V] G [G]	[G] L [L]	[L] V [M]
IPNS-S.lactam	[L] V [L]	[L] V [L]	[A] A [A]	[G] G [G]	[L] V [M]
IPNS-Flavob	[L] A V [L]	[L] A V [L]	[V] G [G]	[G] G [G]	[V] [V]
IPNS-S.gris	[L] A V [L]	[L] A V [L]	[V] G [G]	[G] G [G]	[V] [V]
IPNS-L.lactamgenus	[L] A V [L]	[L] A V [L]	[V] G [G]	[G] G [G]	[V] [V]
IPNS_STRJU	[L] V [L]	[L] V [L]	[V] G A [G]	[G] G [G]	[L] V [M]
IPNS-S.calt	[L] A V [M]	[L] A V [M]	[A] A [A]	[G] G [G]	[L] V [M]
DAOCS-S.clav	[L] G [L]	[L] G [L]	[V] V [V]	[A] A [A]	[L] M A [M]
DACS-S.clav	[L] A [V]	[L] A [V]	[V] V [V]	[A] A [A]	[L] M A [M]
DACS-S.lactam	[L] G [V]	[L] G [V]	[V] V [V]	[A] A [A]	[L] M A [M]
DAOCS-Acrem	[V] G [L]	[V] G [L]	[V] V [V]	[V] A [A]	[L] M G [M]
DACS-L.lactamgenus	[L] G [L]	[L] G [L]	[V] V [V]	[G] A [A]	[W] W [W]
DACS-L.lactamgenus_1	[L] G [V]	[L] G [V]	[V] V [V]	[G] A [A]	[W] W [W]
Consensus	[I]	[I]	[I]	[I]	[I]
Identity	[I]	[I]	[I]	[I]	[I]

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Page 6

IPNS_E.nidulans	+	230	+	250	+	270	+	290
IPNS_S.clav	V	V	AAG	T A	G	L I	E G	R A
IPNS_S.anul	V	V	V G	T I	V	L I	E G	R A
IPNS_S.lactam	V	V	V G	T L	G	L I	E G	R A
IPNS_Flavob	V	V	V G	T I	G	L I	E G	R A
IPNS_S.gris	V	V	V G	T L	G	L I	E G	R A
IPNS_L.lactamgenus	V	V	V G	T I	G	L I	E G	R A
IPNS_STRJU	V	V	V A G	T L	G	L I	E G	R A
IPNS_S.catt	V	V	V A G	T L	G	L I	E G	R A
DAOCS_S.clav	V	V	V A G	T L	G	L I	E G	R A
DACS_S.clav	V	V	V A G	T L	G	L I	E G	R A
DACS_S.lactam	V	V	V A G	T L	G	L I	E G	R A
DAOCS_Acrem	V	V	V A G	T L	G	L I	E G	R A
DACS_I.lactamgenus	V	V	V A G	T L	G	L I	E G	R A
DACS_S.lactamgenus_1	V	V	V A G	T L	G	L I	E G	R A
Consensus	V	V	V A G	T L	G	L I	E G	R A
Identity	V	V	V A G	T L	G	L I	E G	R A

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IPNS-E.nidulans  
IPNS-S.clav  
IPNS-S.sanul  
IPNS-S.laclam  
IPNS-Flavob  
IPNS-S.gris  
IPNS-L.lactamgenus  
IPNS\_STRJU  
IPNS-S.cat  
DAOCS-S.clav  
DACS-S.clav  
DACS-S.laclam  
DAOCS-Acrem  
DACS-L.lactamgenus  
DACS-L.lactamgenus\_1  
Consensus  
Identity

330  
[1A]

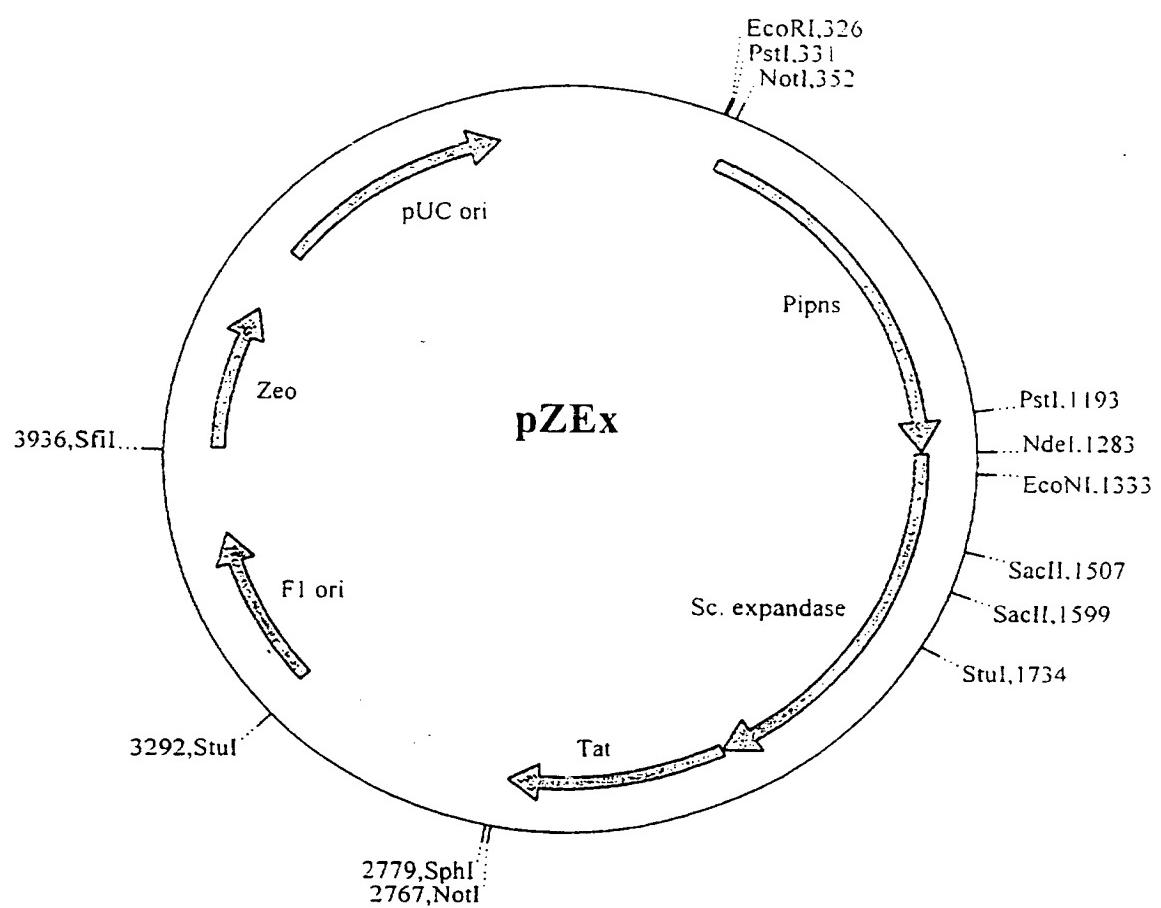


FIG. 2

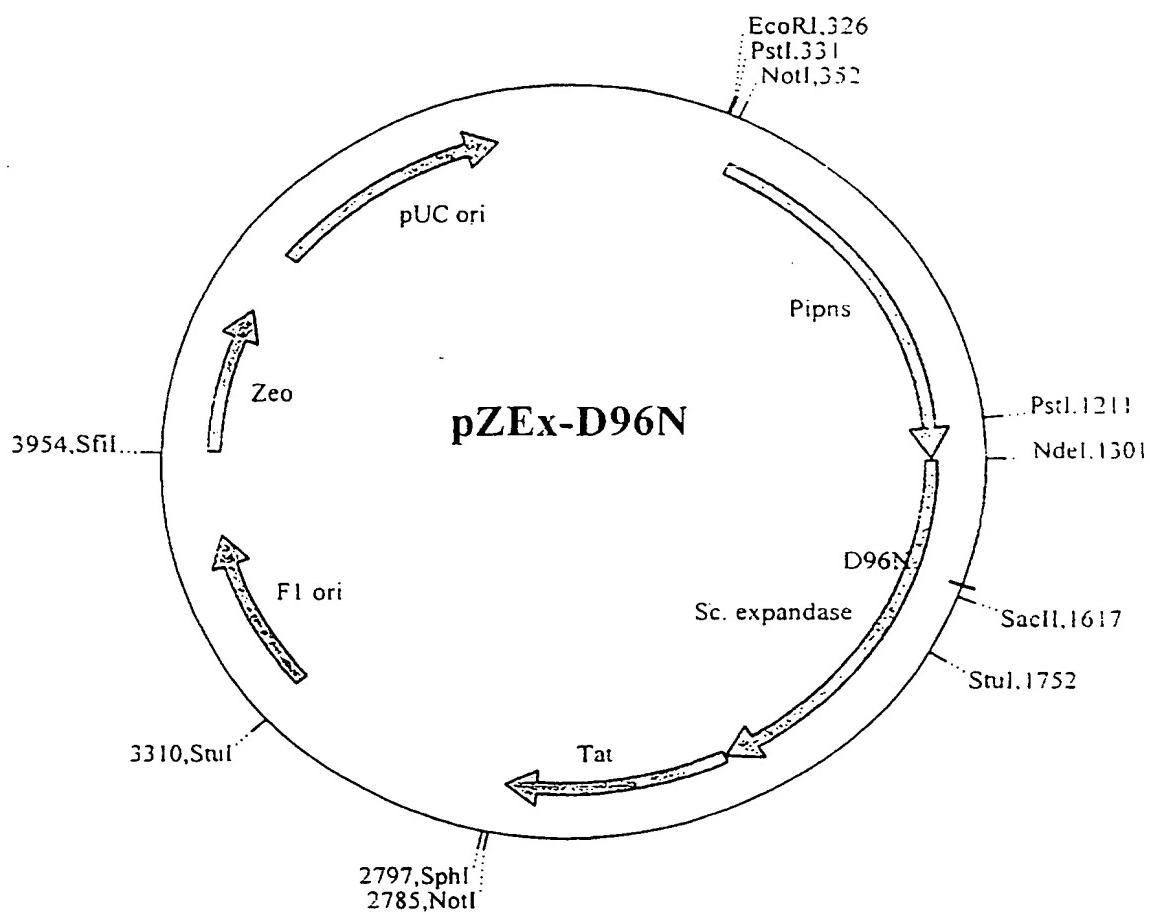


FIG. 3

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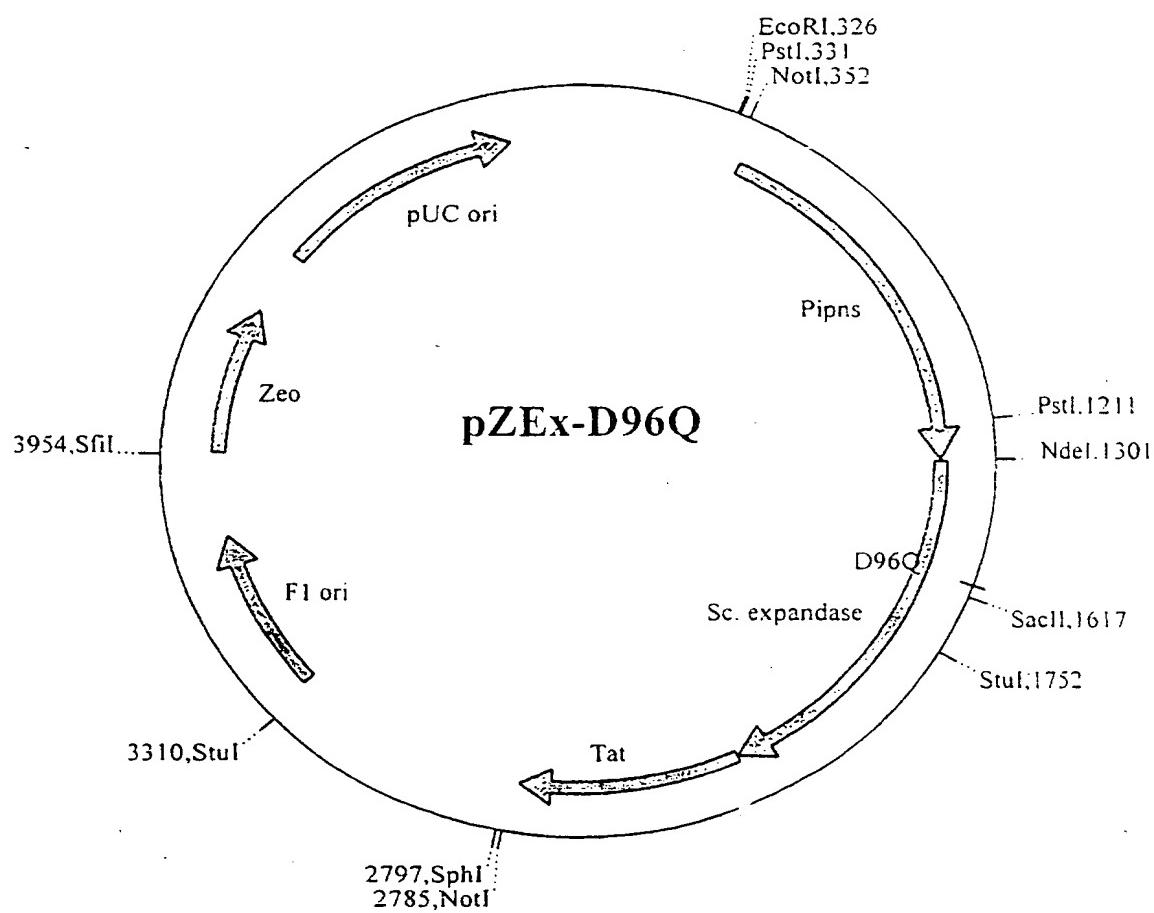


FIG. 4

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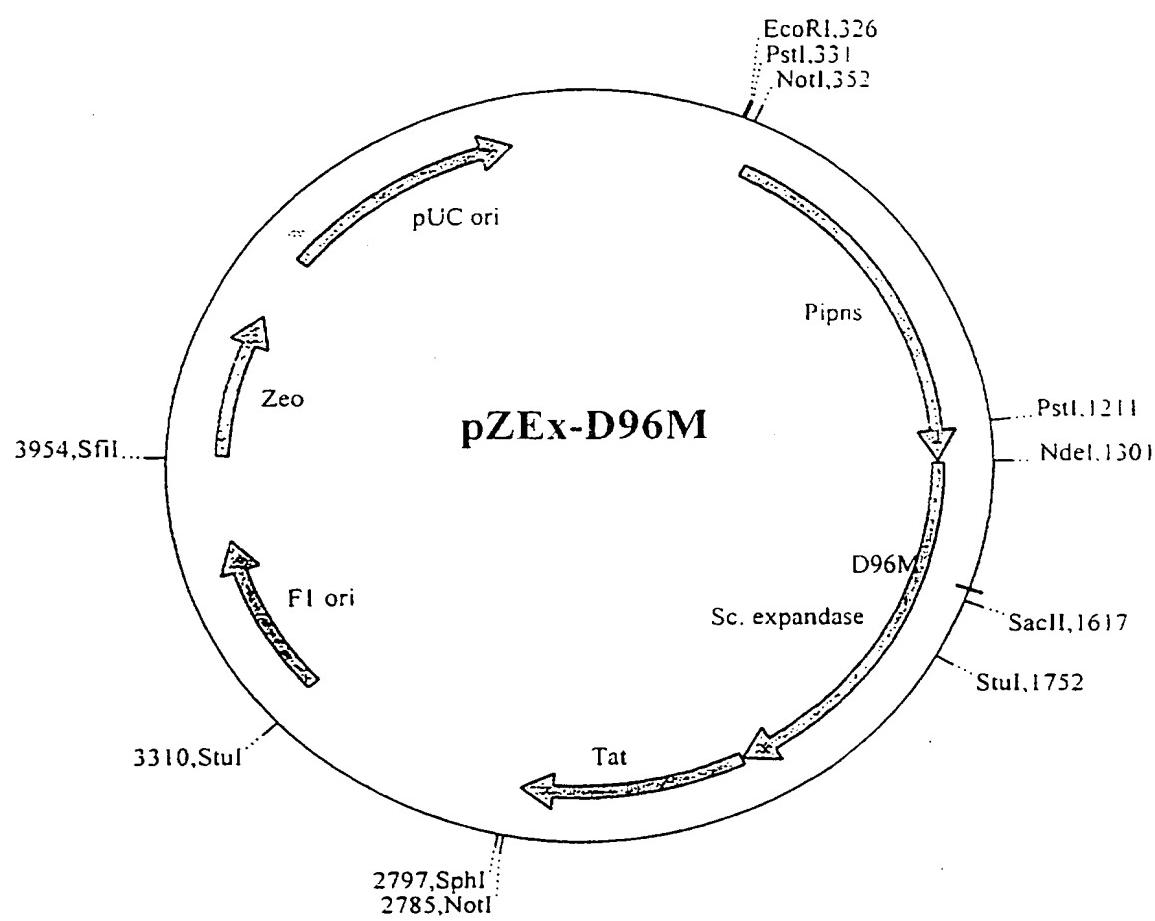


FIG. 5

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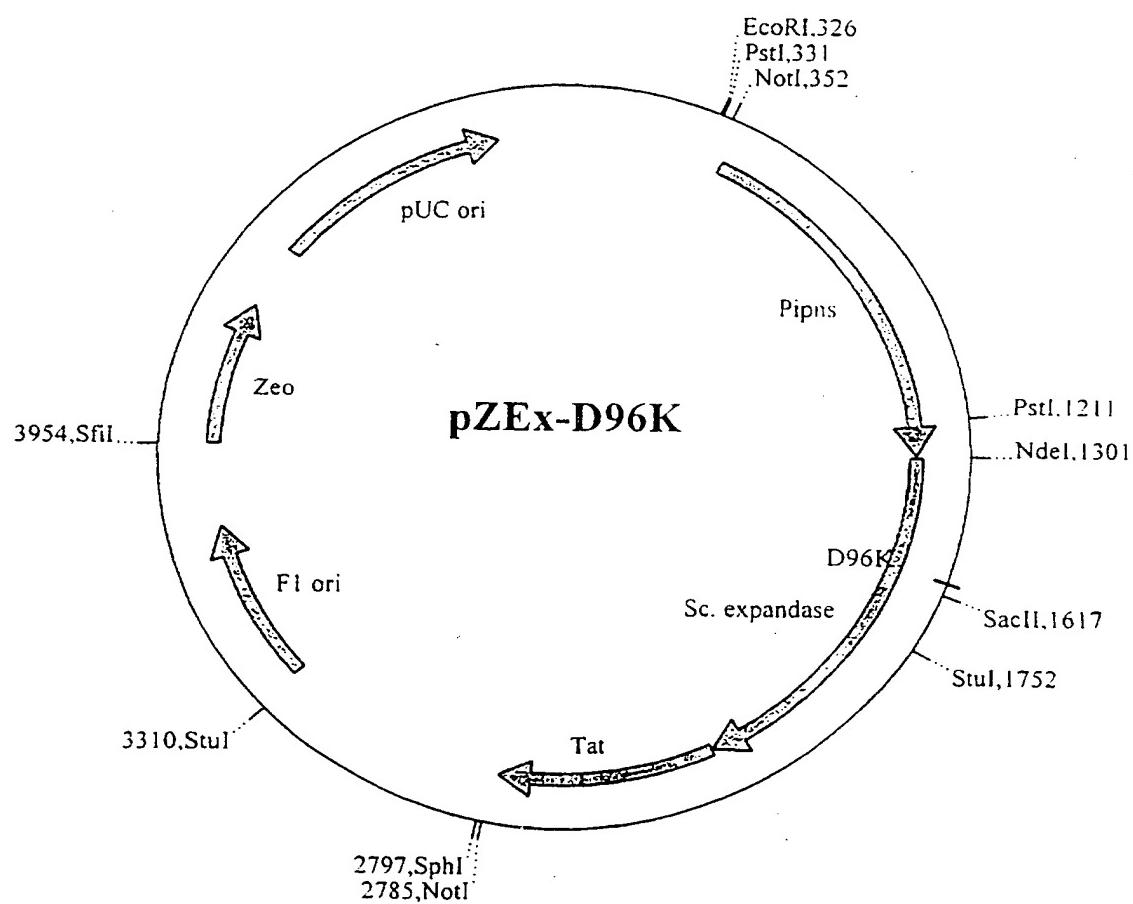


FIG. 6

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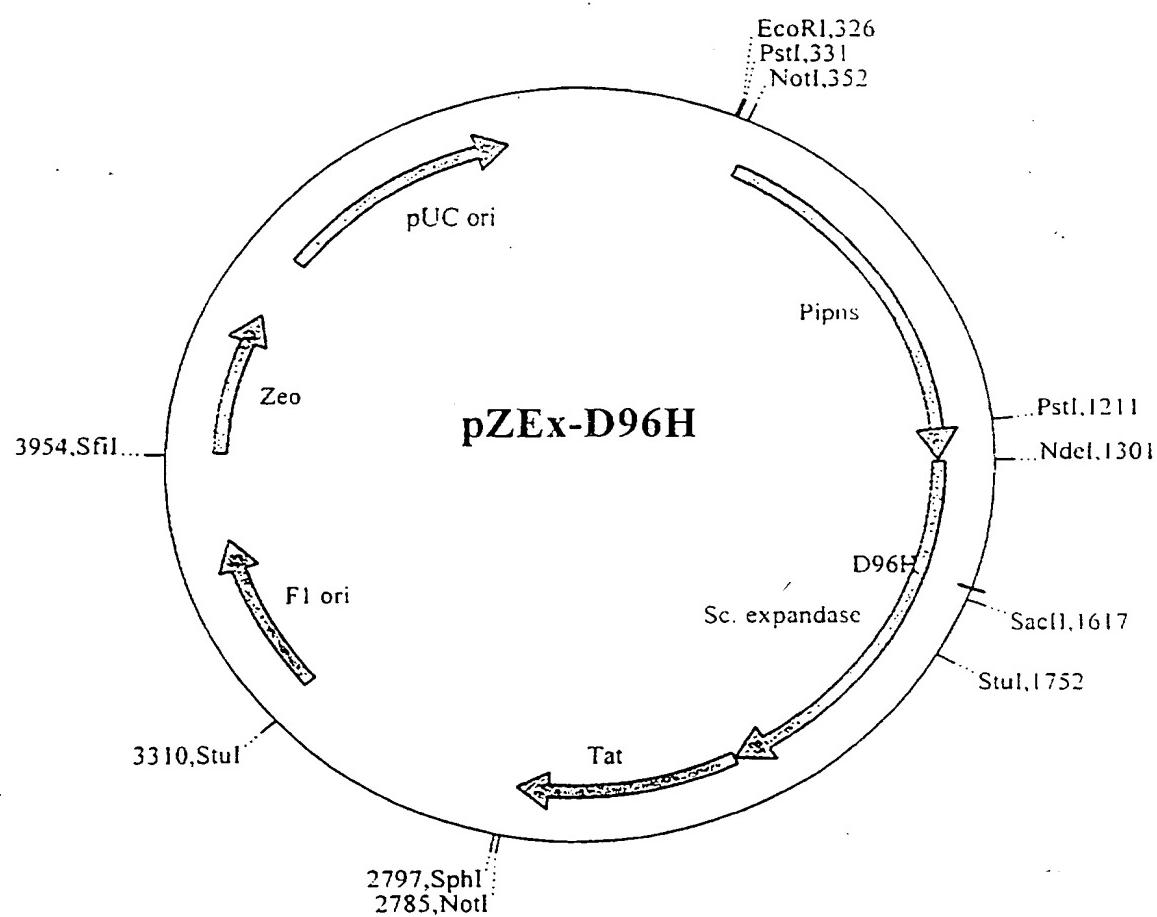


FIG. 7



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 97/03879

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP 0341892 A	15-11-89	US 5070020 A		03-12-91
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		DK 217889 A		13-11-89
		HU 209581 B		29-08-94
		JP 2027984 A		30-01-90
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EP 0366354 A	02-05-90	US 5082772 A		21-01-92
		DE 68925338 D		15-02-96
		DE 68925338 T		05-06-96
		DK 521489 A		25-04-90
		ES 2082787 T		01-04-96
		IL 92079 A		26-08-94
		JP 2242675 A		27-09-90
<hr/>				
WO 9720053 A	05-06-97	AU 1097297 A		19-06-97
<hr/>				





## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 15/52, 9/00, C12P 35/02, C12N 1/15</b>		A3	(11) International Publication Number: <b>WO 98/02551</b> (43) International Publication Date: 22 January 1998 (22.01.98)
(21) International Application Number: <b>PCT/EP97/03879</b> (22) International Filing Date: 15 July 1997 (15.07.97)  (30) Priority Data: 96201988.1 16 July 1996 (16.07.96) EP (34) Countries for which the regional or international application was filed: AT et al.		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW. ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(71) Applicant (for all designated States except US): GIST-BROCADES B.V. [NL/NL]; Wateringseweg 1, P.O. Box 1, NL-2600 MA Delft (NL).  (72) Inventors; and (75) Inventors/Applicants (for US only): BOVENBERG, Roelof, Ary, Lans [NL/NL]; 's-Gravenweg 121, NL-3062 ZD Rotterdam (NL). VAN DER LAAN, Jan, Metske [NL/NL]; Leursebaan 364, NL-4839 AP Breda (NL). KERKMAN, Richard [NL/NL]; Koninginneweg 12, NL-2042 NL Zandvoort (NL). NIEBOER, Maarten [NL/NL]; Gerberasingel 112, NL-2651 XZ Berkel en Rodenrijs (NL).  (74) Agents: VISSER-LUIRINK, Gesina et al.; Gist-Brocades N.V., Patents and Trademarks Dept., Wateringseweg 1, P.O. Box 1, NL-2600 MA Delft (NL).		<p>Published  <i>With international search report.  Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p> <p>(88) Date of publication of the international search report: 19 February 1998 (19.02.98)</p>	

(54) Title: PROCESS FOR THE PRODUCTION OF ADIPOYL CEPHALOSPORINS

## (57) Abstract

An improved process for the preparation of adipoyl cephalosporins via enzymatic ring expansion of adipoyl-6-aminopenicillanic acid, using a *Penicillium chrysogenum* transformant strain expressing modified expandase enzyme.

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 97/03879

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC 6	C12N15/52	C12N9/00	C12P35/02	C12N1/15
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According to International Patent Classification(IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CANTWELL C ET AL: "ISOLATION OF DEACETOXYCEPHALOSPORIN C FROM FERMENTATION BROTHS OF PENICILLIUM-CHRYSOGENUM TRANSFORMANTS CONSTRUCTION OF A NEW FUNGAL BIOSYNTHETIC PATHWAY" PROC R SOC LOND SER B BIOL SCI, 248 (1323). 1992. 283-289., XP002049879 cited in the application see the whole document ---	1-8
A	EP 0 341 892 A (LILLY CO ELI) 15 November 1989 cited in the application see claims 1-27 ---	1-8
A	EP 0 366 354 A (LILLY CO ELI) 2 May 1990 cited in the application see claims 1-10 ---	1-8
		-/-



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Patent family members are listed in annex.

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1

Date of the actual completion of the international search

11 December 1997

Date of mailing of the international search report

14/01/1998

Name and mailing address of the ISA

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NL - 2280 HV Rijswijk  
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Fax: (+31-70) 340-3016

Authorized officer

Gurdjian, D

## INTERNATIONAL SEARCH REPORT

Int'l. Application No

PCT/EP 97/03879

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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